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Clustering of Nicotinic Acetylcholine Receptors: From the Neuromuscular Junction to Interneuronal Synapses

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Abstract

Fast and accurate synaptic transmission requires high-density accumulation of neurotransmitter receptors in the postsynaptic membrane. During development of the neuromuscular junction, clustering of acetylcholine receptors (AChR) is one of the first signs of postsynaptic specialization and is induced by nerve-released agrin. Recent studies have revealed that different mechanisms regulate assembly vs stabilization of AChR clusters and of the postsynaptic apparatus. MuSK, a receptor tyrosine kinase and component of the agrin receptor, and rapsyn, an AChR-associated anchoring protein, play crucial roles in the postsynaptic assembly. Once formed, AChR clusters and the postsynaptic membrane are stabilized by components of the dystrophin/utrophin glycoprotein complex, some of which also direct aspects of synaptic maturation such as formation of postjunctional folds. Nicotinic receptors are also expressed across the peripheral and central nervous system (PNS/CNS). These receptors are localized not only at the pre- but also at the postsynaptic sites where they carry out major synaptic transmission. In neurons, they are found as clusters at synaptic or extrasynaptic sites, suggesting that different mechanisms might underlie this specific localization of nicotinic receptors. This review summarizes the current knowledge about formation and stabilization of the postsynaptic apparatus at the neuromuscular junction and extends this to explore the synaptic structures of interneuronal cholinergic synapses.

Index Entries: Clustering; neuromuscular junction; cholinergic; synapse; synaptogenesis; agrin; nicotinic receptors; postsynaptic density.

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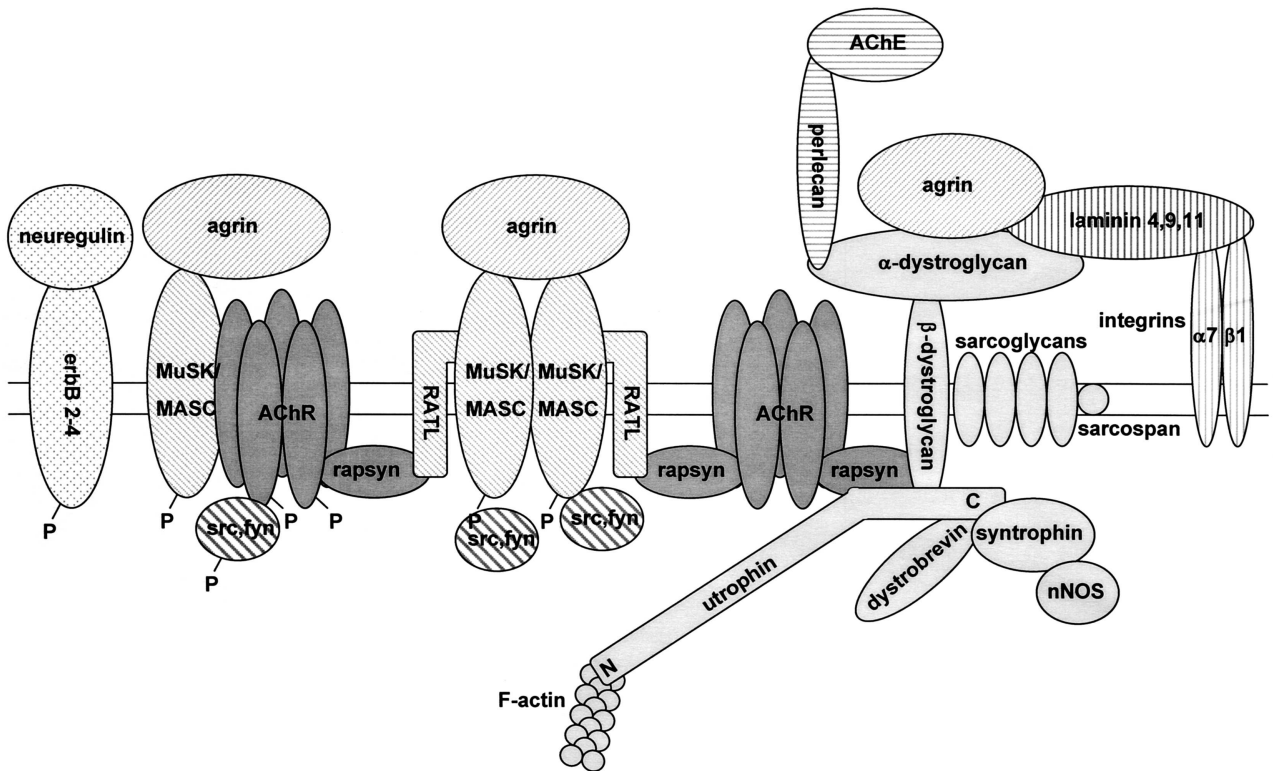


Fig. 1. The postsynaptic apparatus at the neuromuscular junction. Postsynaptic proteins and their interactions are illustrated. For simplicity, not all isoforms of synaptically located proteins are shown. Similarly, segregation of isoforms between the crests and troughs of postjunctional folds is neglected. p, tyrosine phosphorylation.

Introduction

Synapses are the points of contact between neurons and their target cells, and mediate cellular communication in the nervous system. At chemical synapses, neurotransmitter molecules are released from synaptic vesicles in the presynaptic nerve terminal and activate their receptors in the opposing postsynaptic membrane. To ensure rapid and reliable synaptic transmission, these receptors must be present at a high density in clusters located precisely opposite sites of neurotransmitter release in the nerve ending. Accordingly, clustering of neurotransmitter receptors is a key aspect of synaptogenesis and occurs early during the formation of both central and peripheral synapses (1).

Much of the current knowledge about clustering of neurotransmitter receptors originates from studies on the vertebrate neuromuscular junction (NMJ). At this peripheral cholinergic synapse, many identified proteins interact to form a postsynaptic apparatus in the muscle, whose hallmark is the accumulation of nicotinic acetylcholine receptors (AChRs) in high density, reaching up to 10,000 molecules/ μm^2 (1; Fig. 1). Neuronal equivalents of this postsynaptic apparatus can be found in synapses in the central nervous system (CNS). One of the best-characterized examples are glutamatergic synapses, where the postsynaptic density contains a plethora of postsynaptic proteins besides glutamate receptors (2).

Several extended reviews about the NMJ have recently appeared (1,3–5), dealing with

all aspects of NMJ formation, maturation, and elimination. Here, we focus largely on one key aspect of synaptogenesis at the NMJ, the clustering of AChRs, and show possible parallels to localization of neuronal nicotinic receptors at interneuronal cholinergic synapses. Recent studies suggest that different proteins direct formation vs maintenance of AChR clusters and the postsynaptic apparatus at the NMJ. Therefore, we divide the description of muscle AChR clustering into two main parts, formation vs stabilization.

The Neuromuscular Junction: Formation of AChR Clusters and the Postsynaptic Apparatus

The high density of postsynaptic AChRs at the adult NMJ is accompanied by a 1000-fold lower level of extrasynaptic AChRs and is the result of three major signaling processes (1). During development, before motoneurons approach muscles, AChRs are diffusely distributed at a density of about 1000 receptors/ μm^2 throughout muscle fibers. First, upon contact with the nerve ending, these pre-existing AChRs are redistributed to form clusters at sites of nerve-muscle contact. Second, muscle nuclei underneath the nerve terminal start to transcribe genes encoding AChRs at elevated levels. Third, extrasynaptic nuclei cease the transcription of AChR genes. Together, these three processes cause the high density of AChRs in the postsynaptic membrane and the virtual absence of AChRs throughout the extrasynaptic plasmalemma. In this review, we largely focus on the first of these processes, the clustering of AChRs.

Agrin and Neuregulin Are Two Key Factors for Formation of the NMJ

During development of the NMJ, AChR clustering is induced by agrin, a key proteoglycan released from the nerve (6). Originally isolated from the electric organ of *Torpedo*

californica based on its ability to cluster AChRs upon addition to cultured myotubes (6), agrin turned out to be the main organizer of the NMJ. Agrin is synthesized by motoneurons, transported along their axons, and released into the extracellular milieu, leading to accumulation in the synaptic basal lamina. In its absence, in agrin-deficient knockout mice, differentiated NMJs fail to form, and nerve-associated clusters of AChRs and other postsynaptic proteins are no longer observed (7). Agrin's crucial role in postsynaptic assembly is further illustrated in microinjected myofibers *in vivo*, where injection of agrin cDNA causes the local formation of ectopic postsynaptic specializations that contain clustered AChRs in association with many other postsynaptic proteins (8,9). Thus, agrin is both necessary and sufficient to induce AChR clustering and formation of the postsynaptic apparatus. Finally, agrin may also play a role, perhaps indirectly, in presynaptic differentiation, because in agrin-deficient animals, motoneurons grow extensively over muscle fibers of the diaphragm without being restricted to a central endplate zone (7). The signaling pathway of agrin in muscle includes the receptor tyrosine kinase (RTK) MuSK as a key element and will be described below.

AChR transcription by myonuclei underneath the nerve terminal is induced at least in part by neuregulin-1 (NRG-1), a protein expressed by motoneurons and skeletal muscle and concentrated at the NMJ (3,10). NRG-1 is a member of the neuregulin family of differentiation factors and was originally isolated as ARIA (AChR-inducing activity) from chick brain extracts, based on its ability to induce AChR gene expression in cultured myotubes (10). As shown by heterozygous recombinant mice lacking one copy of the NRG-1 gene, NRG-1 is indeed necessary to maintain the high synaptic density of AChRs *in vivo* (11). NRG-1 acts by activating ErbBs (ErbB2-4), members of the EGF receptor family of RTKs, which are concentrated at the NMJ (12,13). ErbB activation in turn triggers a generic signaling cascade including kinases such as Ras,

Raf, MAP kinases, and phosphatidylinositol-3-kinase, as well as the adapter protein Shc and the tyrosine phosphatase SHP2 (14–17). Together, activation of these signaling intermediates culminates in enhanced transcription of AChR genes in synaptic nuclei. This transcription is thought to be mediated by binding of members of the ets family of transcription factors, such as GABP α and GABP β , to N-box motifs in the regulatory regions of the AChR subunit genes (18,19).

Interestingly, recent studies indicate that the agrin and NRG-1 signaling pathways, which both contribute to the high density of AChRs in the postsynaptic membrane, are linked, and that agrin acts upstream of NRG-1 in the formation of the NMJ. Thus, besides clustering of pre-existing AChRs, agrin can induce AChR gene expression in cultured myotubes (20) and in injected myofibers in vivo (21,22). Furthermore, NRG-1 binds to agrin in vitro, and agrin-induced AChR gene transcription is dependent on signaling through ErbB2 in cultured myotubes (23). As ErbBs are no longer clustered at mutant NMJs of mice lacking agrin (7), these observations suggest that agrin acts upstream of NRG-1 in postsynaptic differentiation, by inducing clustering of NRG-1 receptors, thereby enabling NRG-1 to locally stimulate AChR gene transcription in subsynaptic nuclei.

MuSK, a Component of the Agrin Receptor

Despite a long-standing interest in the mechanisms of agrin signaling and postsynaptic assembly, many steps in agrin-induced clustering of pre-existing AChRs remain uncertain. It is clear, however, that neurally released agrin activates a receptor in the muscle membrane, which initiates a signaling cascade that ultimately leads to clustering and anchoring of AChRs via intracellular and AChR-associated signaling and anchoring proteins (1,6). A key component of the functional agrin receptor is the RTK, MuSK. The crucial role of this RTK is best illustrated by MuSK-deficient mice, which lack differentiated NMJs, clusters of AChRs

and other postsynaptic proteins, and thus display a similar phenotype as agrin $-/-$ mice (24). Moreover, cultured MuSK $-/-$ myotubes fail to respond to agrin, whereas agrin triggers a rapid tyrosine phosphorylation of MuSK when added to wild-type muscle cells, further showing that MuSK is a part of the agrin receptor (25).

In analogy to certain other RTKs that require co-receptors to bind to their extracellular ligands, MuSK itself is unable to directly bind to agrin (25). This observation has led to the proposal that other proteins of myotubes (designated MASC, for myotube-associated specificity component) associate with MuSK to form a fully functional agrin receptor. The nature of MASC, however, has remained largely elusive, although MASC most likely interacts with the first immunoglobulin-like domain in the extracellular part of MuSK (26). Based on its tight binding to agrin, α -dystroglycan, which is a component of the dystrophin/utrophin glycoprotein complex (D/UGC), was originally proposed to form part of a functional agrin receptor, thus being a candidate for MASC. Subsequent studies have shown, however, that agrin fragments that do not bind to α -dystroglycan still induce AChR clustering and activate MuSK (27–29), and that agrin-induced MuSK activation is normal in antisense-treated myotubes that express low levels of α -dystroglycan (30). Thus, α -dystroglycan does not seem to be the MASC co-receptor for MuSK.

Another important question is how, following stimulation by agrin, MuSK transmits a signal into myotubes. By analogy with other RTKs, MuSK signaling is expected to involve binding of signal-transducing molecules to phosphorylated tyrosine residues in the cytoplasmic domain of agrin-activated MuSK. Following treatment with agrin, MuSK indeed undergoes autophosphorylation, probably on several cytoplasmic tyrosine residues (31). By mutational analysis, two of these tyrosines were found to be required for agrin-induced AChR clustering in myotubes. One tyrosine is located in the activation loop of the kinase

domain, showing that the kinase activity of MUSK is necessary for its responsiveness to agrin (26,32). In addition, a juxtamembrane tyrosine is required as well and can, along with the sequences surrounding it, actually confer agrin responsiveness when expressed in otherwise inactive MuSK-TrkA chimeras (32). As the juxtamembrane tyrosine occurs within an NPXY sequence context, the consensus binding site for Shc and related adapter proteins, one or several Shc-like proteins are likely to be involved as downstream mediators in the agrin signaling pathway.

Rapsyn, an AChR Clustering Protein

A central player in the agrin signaling pathway downstream of MuSK is rapsyn. This protein was originally identified as a 43 kDa protein in Torpedo synaptic membranes based on its close association with AChRs (33). Rapsyn is necessary for clustering of AChRs and many aspects of postsynaptic differentiation in muscle. Thus, rapsyn-deficient mice lack aggregates of AChRs, dystroglycan, utrophin and ErbB receptors, and cultured rapsyn $-/-$ myotubes do not form AChR clusters spontaneously nor even in response to agrin (34). Because MuSK is still localized synaptically at mutant NMJs in these mice and is tyrosine-phosphorylated by agrin in rapsyn $-/-$ myotubes, rapsyn clearly acts downstream of MuSK (35).

The mechanism of rapsyn-dependent clustering of AChRs has been extensively analyzed by protein expression in heterologous cells. In transfected nonmuscle cells, rapsyn causes clustering of itself, and, upon co-expression, of AChRs and certain other postsynaptic proteins such as MuSK or dystroglycan. This suggests that rapsyn is a general mediator of protein clustering (36–38). Rapsyn has a unique protein structure in which an N-terminal myristoylation site is followed by eight tetratricopeptide repeats (TPR), a coiled-coil motif, a ring zinc-finger motif, and a serine phosphorylation site (reviewed in ref. 39). By heterologous

expression, some of these domains were found to mediate distinct aspects of rapsyn functioning. First, N-terminal myristoylation mediates targeting of rapsyn to the plasma membrane (40,41). Second, at least two of the eight TPRs are necessary and sufficient for self-association of rapsyn as clusters in transfected cells, whereas, third, the coiled-coil motif is required for co-clustering of AChRs in rapsyn aggregates (40,42). Furthermore, rapsyn clusters each of the AChR subunits in heterologous cells and requires the intracellular portion of the subunit for this, as shown by the AChR α subunit (43). Together, these recent studies suggest that rapsyn interacts with the cytoplasmic moieties of all subunits of the AChR and that rapsyn self-association and rapsyn-induced AChR clustering are mediated by different domains in rapsyn.

Rapsyn also causes clustering of MuSK and dystroglycan upon co-expression in QT-6 cells, and biochemical evidence shows that rapsyn directly binds to the cytoplasmic portion of β -dystroglycan (44). In the case of MuSK, the interaction, unexpectedly, does not depend on the cytoplasmic tail of MuSK, but involves the fourth immunoglobulin-like domain in MuSK's exodomain, leading to the concept that a rapsyn-associated transmembrane linker protein (RATL) links rapsyn to the exodomain of MuSK (Fig. 1; 26,35). This linker, whose identity remains unsolved, may recruit rapsyn and its associated proteins to MuSK and thereby play a crucial role in agrin signaling.

These results are largely based on studies performed using heterologous cell systems and may not be entirely applicable to myotubes, where clustering is regulated in different and/or additional ways: in myotubes, efficient AChR clustering requires agrin and MuSK, and the clusters are much larger. In myotubes, indeed, the expression level of rapsyn is an important parameter that determines its ability to mediate agrin-induced AChR clustering. Endogenous rapsyn normally occurs in about a 1:1 stoichiometry with the AChR in muscle (45). Upon overexpression of rapsyn in wild-type or rapsyn $-/-$

myotubes, fewer spontaneous and agrin-induced AChR clusters are seen than in cells expressing physiological levels of rapsyn (46,47). This effect appears to originate from intracellular accumulation of AChRs in the case of high rapsyn expression, implying a role of rapsyn in trafficking of AChRs to the plasma membrane (48). Consistent with such a role, rapsyn was recently colocalized with AChRs in post-Golgi transport vesicles of Torpedo electric organ (49). Another example illustrating the differences between heterologous and muscle cells is the zinc ring finger domain of rapsyn, which is dispensable for rapsyn-induced AChR clustering in heterologous cells but not for spontaneous AChR aggregation in myotubes (42,50). These studies suggest that in myotubes, rapsyn functioning depends on interaction with other, as yet unidentified muscle-specific proteins.

Downstream Agrin Signaling: a Role of AChR β Phosphorylation and Src-Family Kinases

Prominent other steps in the agrin signaling pathway downstream of MuSK are tyrosine-phosphorylation of the AChR and a link of the AChR to the cytoskeleton. Four recent studies now show that phosphorylation of the AChR β subunit and Src-family kinases play a role in these processes and hence for formation and stabilization of AChR clusters (51–54).

In myotubes in culture, agrin induces tyrosine phosphorylation of the AChR β and δ subunits as well as a link of the AChR to the cytoskeleton, as shown by AChR extraction assays (52,55, 56). Also, developing NMJs in vivo contain phosphotyrosine-proteins that codistribute with AChRs, and AChRs at these junctions are progressively stabilized, presumably through a link to cytoskeletal elements (57,58). In agrin-treated myotubes, AChR β and δ phosphorylation occur early before clusters are visible (52); β phosphorylation is paralleled by decreased extractability of the AChR, which also precedes clustering (55). Several

strategies have been applied to determine whether the AChR phosphorylation induced by agrin plays a role in AChR clustering and cytoskeletal linkage. For example, inhibitors of tyrosine kinases, such as herbimycin and staurosporine, block both agrin-induced AChR β and δ phosphorylation and clustering (52,56, 59). Conversely, increasing AChR phosphorylation by treatment of myotubes with pervanadate causes an increased cytoskeletal link of AChRs (60). Most importantly, when mutated AChRs, lacking cytoplasmic tyrosine residues in their β subunits, are expressed in transfected myotubes, they are still able to form agrin-induced clusters (61), but the extent of clustering and their cytoskeletal linkage are clearly reduced when compared to wild-type AChRs (51). Thus, agrin-induced phosphorylation of the AChR β subunit regulates the link of the AChR to the cytoskeleton and contributes to clustering of AChRs.

Another issue is which kinase(s) causes phosphorylation of the AChR subunits in response to agrin and what role such a kinase plays in agrin-induced AChR clustering. MuSK is unlikely to directly phosphorylate the AChR, as illustrated in rapsyn $-/-$ myotubes and staurosporine-treated wild-type myotubes, in which agrin activates MuSK, whereas AChR β and δ phosphorylation and clustering are blocked (35,52,62). These studies strongly imply the existence of some other kinase(s) downstream of MuSK. Accumulating evidence suggests that this kinase(s) is a member of the Src-family: first, Src and Fyn are associated with AChRs in myotubes (63), whereas Fyn and Fyk are major kinases in Torpedo electric organ and associate with AChRs in this tissue (64,65). Second, Src can phosphorylate AChR β fusion proteins containing the tyrosine residue that is phosphorylated in agrin-treated myotubes (51,63). Third and most importantly, AChR-bound Src family members are activated and tyrosine-phosphorylated in agrin-treated myotubes, and this activation is abolished by staurosporine and not observed in rapsyn-deficient myotubes, and thus correlates

highly with AChR clustering (52). Fourth, similarly, each of the Src family kinases Src, Fyn and Fyk can interact with rapsyn in transfected fibroblasts, leading to an activation of these kinases and phosphorylation of cotransfected AChRs (66). Fifth, Src and Fyn form a complex with MuSK in myotubes, are able to phosphorylate MuSK, and their binding to MuSK depends on their SH2 domain and is increased by agrin (53). Sixth, of significance, in myotubes, the agrin-induced linkage of AChRs to the cytoskeleton is blocked by a specific inhibitor of the Src family, PP1, and agrin-induced AChR clustering is reduced by expression of dominant-negative, kinase-inactive Src, resulting in AChR aggregates that are very small (53). Finally, by using knockout mice, the role of Src and Fyn in postsynaptic differentiation, AChR clustering and phosphorylation was directly tested (54). In mice lacking Src and Fyn, all aspects of synaptic development examined such as innervation, AChR gene expression, and clustering of postsynaptic proteins are normal until around birth, by which time these animals die (54). In cultured Src-Fyn-deficient myotubes derived from these mice, agrin causes normal AChR clustering and AChR β phosphorylation, and the Src-family kinase Yes is upregulated. Interestingly, following withdrawal of agrin, agrin-induced clusters of AChRs are dramatically less stable in myotubes lacking Src and Fyn than in wild-type cells (54).

Together, these recent studies show that kinases of the Src family play a role in agrin signaling: they interact with MuSK in an agrin-dependent manner and are activated by agrin locally, when bound to the AChR. Further, their functioning is required to link the AChR to the cytoskeleton and for growth of AChR clusters. Src and Fyn themselves, however, are dispensable for AChR clustering and phosphorylation, although, in their absence, Yes may compensate. Nonetheless, Src and Fyn are clearly required to stabilize agrin-induced AChR aggregates, showing that the mechanisms for formation vs stabilization of these clusters are different.

Protein Interactions and Steps in Formation of the Postsynaptic Apparatus: Evidence for Postsynaptic Pre-Assembly

Src-family kinases interact both with MuSK and the AChR in myotubes. In both cases, some interaction occurs before addition of agrin, thus revealing the existence of pre-assembled protein complexes. However, these interactions appear to reflect two distinct pools of Src-family kinases that respond differently to treatment with agrin. Thus, agrin causes Src members to increasingly bind to MuSK (53), but activates AChR-associated Src kinases without affecting their interaction with AChRs (52). Furthermore, agrin induces MuSK phosphorylation independently of rapsyn (35), implying that Src-mediated MuSK phosphorylation (53) does not require rapsyn. In contrast, activation of AChR-bound Src members by agrin critically depends on rapsyn (52). Therefore, there is increasing evidence for the existence of two independent pre-assembled signaling complexes in myotubes, a MuSK-bound complex and an AChR-bound complex (Fig. 2).

How do these complexes contribute to postsynaptic assembly? The available evidence suggests that the MuSK-bound complex acts as a primary synaptic scaffold and is clustered first due to agrin; this scaffold then recruits pre-assembled AChR complexes via rapsyn (Fig. 2). These steps of postsynaptic assembly are based, first, on the observation that MuSK is the only identified postsynaptic protein to still be clustered at mutant synapses of rapsyn-deficient mice (35). In addition, when expressed ectopically in injected muscle fibers *in vivo*, a constitutively active form of MuSK is able to organize postsynaptic specializations containing AChRs in association with most other postsynaptic components (67). Second, rapsyn induces aggregation of AChRs, dystroglycan, MuSK, and of itself when expressed in heterologous cells (36–38), suggesting that rapsyn recruits other postsynaptic components to the MuSK scaffold. Third, in cultured

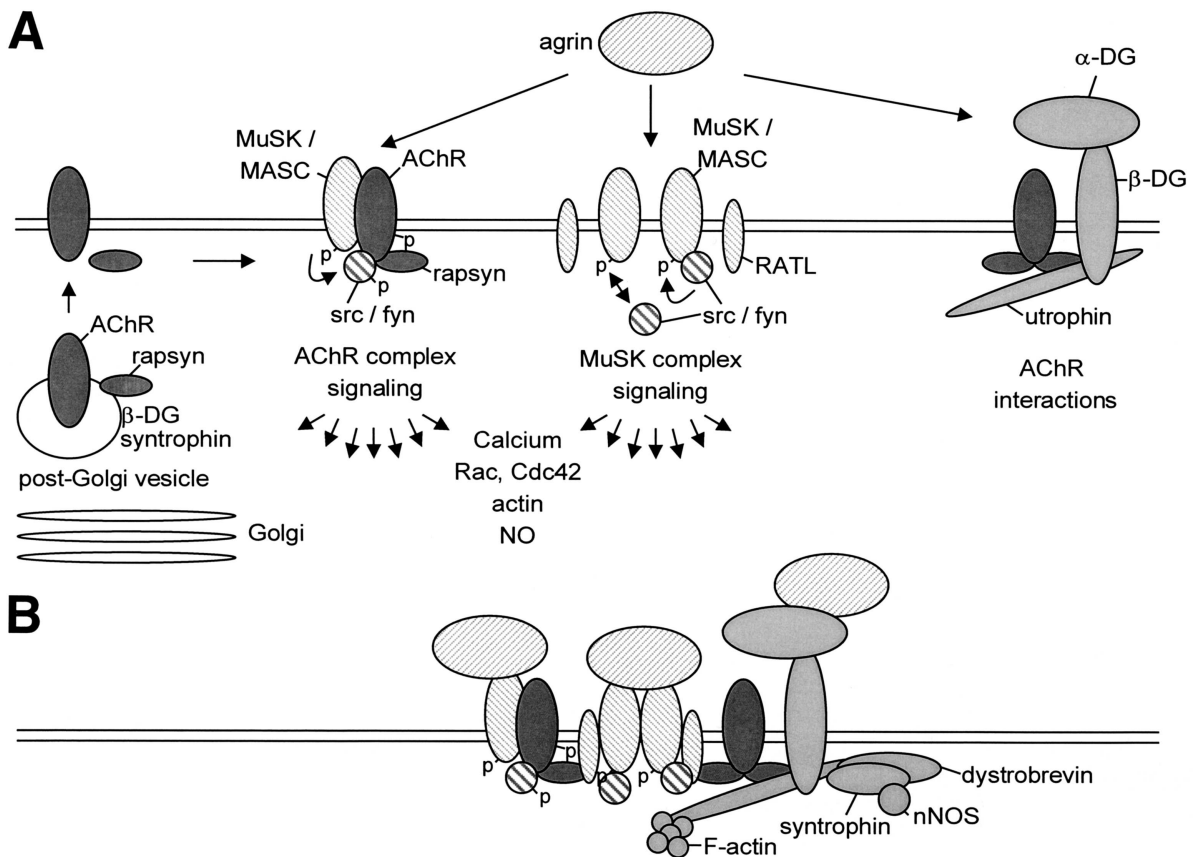


Fig. 2. Steps in the assembly of the postsynaptic membrane at the NMJ. **(A)** In the absence of nerve-derived agrin, at least two pre-assembled signaling complexes exist in the muscle membrane, an AChR complex and a MuSK complex. Assembly of these complexes may in part occur in the secretory pathway, since some of their components colocalize in post-Golgi vesicles. Two main local signaling events leading to AChR clustering in response to agrin are depicted. In the MuSK complex, which may act as a primary synaptic scaffold, agrin causes rapid activation of MuSK and recruitment of Src and Fyn to MuSK, leading to MuSK phosphorylation. In the AChR complex, agrin activates MuSK rapidly; in a rapsyn-dependent way, AChR-bound Src and Fyn are then autophosphorylated, and AChR β and δ subunits are tyrosine-phosphorylated. Further elements involved are Calcium fluxes, activation of Rac and Cdc42, NO and actin filaments, which are all required for formation of AChR clusters. **(B)** After prolonged treatment with agrin, clusters of postsynaptic proteins are formed, at least in part by a rapsyn-dependent linkage of the pre-assembled AChR complexes shown in **(A)** to the MuSK complex/primary synaptic scaffold. α -DG, α -dystroglycan; p, tyrosine phosphorylation.

myotubes, AChRs interact with rapsyn, MuSK, Src-family kinases, dystroglycan, and utrophin independently of agrin, showing that diffusely distributed AChRs can pre-assemble pools of postsynaptic components including rapsyn (68). Such complexes associate with MuSK—presumably the MuSK primary

synaptic scaffold—in response to agrin, leading to a rapsyn-dependent increase in the AChR-MuSK interaction, whereas other AChR-protein interactions are not affected by agrin (Fig. 2; 68). Fourth, intracellular AChRs of Torpedo electric organ are colocalized with rapsyn, dystroglycan, and syntrophin in post-

Golgi transport vesicles, implying that some formation of pre-assembled AChR complexes may occur intracellularly (69).

Taken together, these studies imply an active functional role of AChRs in synaptic assembly. Consistent with this idea, agrin does not induce clusters of acetylcholinesterase in myotubes where AChRs were downregulated with anti-AChR antibodies (70). Further, agrin-induced clustering of a full set of postsynaptic markers requires the presence of AChRs in myotubes, as shown recently in derivatives of C2 myotubes expressing low levels of AChRs, as well as by using C2 myotubes in which surface AChRs were downregulated by anti-AChR antibodies (71a). In particular, after downregulating AChRs by treating with antibodies, clusters of rapsyn were not produced in response to agrin (71a). Thus, accumulating evidence suggests that AChRs play an active and early role in postsynaptic assembly, at least in part by forming complexes with other postsynaptic components, which are pre-assembled prior to the treatment of agrin (Fig. 2).

Other Signaling Intermediates in Agrin-Induced AChR Clustering: Calcium, GTPases, and nNOS

Agrin-induced postsynaptic assembly (Fig. 2) is likely to be regulated by additional signaling intermediates. Calcium fluxes are required for AChR clustering, as shown by inhibition of agrin-induced AChR clustering by the fast calcium chelator, BAPTA-AM (72). Furthermore, Rac and Cdc42 are necessary for agrin-induced clustering of AChRs in myotubes, because dominant-negative forms of these small GTPases inhibit aggregation of AChRs in transfected myotubes (73). Rac and Cdc42 are known from other systems to control actin polymerization, for example, causing focal reorganization of the actin cytoskeleton in response to extracellular cues. Indeed, latrunculin A inhibits agrin-induced AChR clustering in cultured *Xenopus* muscle cells, confirming the importance of F-actin assembly in AChR

aggregation (74). It is unclear, however, how F-actin acts in AChR clustering. A synaptic component of the D/UGC, utrophin, binds to actin but is dispensable for AChR clustering (75), implying that actin acts through other binding proteins.

Another signaling mediator that was recently recognized to play a role in agrin-induced AChR aggregation is nitric oxide, NO (76). Neuronal nitric oxide synthetase, nNOS, was known for several years to be concentrated at NMJs, through binding to isoforms of syntrophin, components of the D/UGC (1). Recently, nNOS was found to be co-clustered with AChRs in agrin-treated cultured myotubes (77), and NO was identified as a downstream mediator in agrin-induced AChR clustering and AChR β phosphorylation, as shown by using NOS inhibitors and NO donors (76). The targets of NO could, in principle, involve Src family kinases, as these are activated by NO in fibroblasts; this idea is attractive because of the activation of Src-family kinases by agrin in myotubes and their role in stabilization of AChR clusters (52,54).

AChR Clustering by Laminin

Besides agrin, a number of other factors stimulate AChR clustering when added to cultured muscle cells. These include laminin (78,79) and VVA-B4, a lectin that binds synapse-specific carbohydrates (80). In addition, basic fibroblast growth factor (bFGF) and heparin-binding growth-associated molecule are also active in clustering when bound to polystyrene beads (81,82). The relevance of many of these factors for AChR clustering and NMJ development *in vivo* is unclear. However, laminin is of particular interest, as it is present synaptically throughout formation of the NMJ and colocalizes with AChR clusters both *in vivo* and *in vitro* (83), suggesting that laminin may provide a supplemental pathway for AChR clustering besides agrin. In the adult, laminin is a major component of the muscle-fibre basal lamina, with distinct synaptic and extrasynaptic isoforms. Laminin molecules are

heterotrimers composed of three chains, α , β , and γ , which exist in multiple forms encoded by separate genes, giving rise to many laminin isoforms. The basal lamina at mature NMJs contains three major laminin forms, laminin-4 ($\alpha 2\beta 2\gamma 1$), laminin-9 ($\alpha 4\beta 2\gamma 1$), and laminin-11 ($\alpha 5\beta 2\gamma 1$) (Fig. 1), whereas laminin-2 ($\alpha 2\beta 1\gamma 1$) is the predominant extrasynaptic isoform found in adult muscle and transiently present at developing NMJs (84). Laminin-1 ($\alpha 1\beta 1\gamma 1$), in turn, is expressed early in skeletal muscle development and around the time of initial synapse formation. A crucial role in neuromuscular synaptogenesis has been shown for the $\beta 2$ chain. In $\beta 2$ -deficient mice, synaptic organization is aberrant: postsynaptic folds are reduced, the number of active zones in the nerve terminal is decreased, and Schwann cells extend processes into the synaptic cleft (85). Schwann cell intrusion originates from the absence of laminin-11, which normally prevents Schwann cell entry by inhibiting growth of their processes (86).

Binding of agrin to laminin appears to form the basis for the localization and tight association of agrin with the muscle basal lamina. The N-terminal portion of the major isoform of agrin in the PNS, designated as the NtA domain (87,88), binds with high affinity to laminin-1, -2, and particularly well to the synaptic laminin-4 (89). Binding occurs through the coiled coil domain of the $\gamma 1$ chain (90,91). This binding may thus contribute to synaptic localization of agrin and suggests that both laminin and agrin act together in synaptic development.

Laminin-1 and merosin (laminin-2/4) cluster AChRs when added to cultured myotubes (78,79,92). The laminin signaling pathway is different from that of agrin and does not involve MuSK or tyrosine phosphorylation of AChRs (78,79). Laminin-induced AChR clustering occurs with a slower time-course and leads to higher densities of AChRs in clusters than in the case of agrin. Moreover, the laminin clustering pathway involves dystroglycan, as shown both in antisense myotube derivatives expressing very low amounts of dystroglycan, and in

myotubes differentiated from dystroglycan $-/-$ embryonic stem cells, where laminin fails to cause AChR aggregation (78,93). Laminin signaling may also involve integrins, because $\alpha 7\beta 1$ integrins are coclustered with AChRs in response to laminin, and as anti- $\alpha 7$ antibodies modulate the AChR clustering ability of laminin (Fig. 1; 94). This is consistent with the postsynaptic localization of several forms of these receptors at endplates, in particular, $\alpha 3$, $\alpha 7$, α_v , and $\beta 1$ (5). Interestingly, integrins are also suggested to act as modulators in agrin signaling, in particular, the $\alpha_v\beta 1$ heterodimer (95), based on antibody inhibition experiments. In addition, cells overexpressing α_v and $\beta 1$ integrin adhere to agrin, revealing a possible binding of agrin to this particular integrin.

Thus, integrins, in particular those containing $\beta 1$, appear to be involved in laminin as well as agrin signaling. Accordingly, besides their differences, agrin and laminin share some of their signaling mechanisms, explaining the additive effects of agrin and laminin on AChR clustering in myotubes (79). Together, these studies indicate that agrin and laminin can act in concert to lead to maximal AChR clustering, which may be important for postsynaptic differentiation at the NMJ in vivo.

Role of Oligosaccharides in AChR Clustering

Carbohydrates that terminate in N-acetylgalactosamine (β GalNAc) are selectively enriched at the NMJ and react with specific lectins such as VVA-B4 (80). Recent studies have shown that several factors that bind to and/or modify oligosaccharides, preferentially complex-type N-linked sugars, can induce AChR clustering in cultured myotubes, thus raising the possibility that such oligosaccharides contribute to AChR aggregation during formation of the NMJ in vivo. These factors include VVA-B4, neuraminidase, and α -galactosidase (80,96,97). While VVA-B4 acts via a pathway independent of MuSK and AChR phosphorylation, possibly via α -dystroglycan (98), neuraminidase increases MuSK

and AChR phosphorylation, indicating that oligosaccharides may be involved both in agrin-dependent and agrin-independent mechanisms of AChR clustering. Of particular interest are VVA-B4-reactive sugars containing β GalNAc. At the NMJ, β GalNAc occurs in the context of a defined oligosaccharide structure, the CT carbohydrate antigen, which contains the disaccharides Gal β 1,3GalNAc and Gal β 1,4GlcNAc as prominent components (99). Both of these disaccharides induce AChR clustering in myotubes as well as MuSK autophosphorylation in vitro, as shown by in vitro phosphorylation assays (99). Interestingly, agrin contains a mucin-like domain to which lactosamine-like saccharides are attached. Fragments of agrin containing the mucin domain stimulate MuSK autophosphorylation in vitro, which can be inhibited by adding Gal β 1,3GalNAc or Gal β 1,4GlcNAc. Finally, these disaccharides bind to agrin and MuSK in vitro (99).

The CT antigen thus can serve a similar function to that proposed for the MuSK coreceptor MASC, since it can bind to agrin and MuSK, and stimulate the MuSK kinase activity. Therefore, the CT carbohydrate may act as MASC. It remains to be determined on which protein the active CT carbohydrates are present, and which protein(s) are recognized by VVA-B4 at NMJs in vivo.

Stabilization of AChR Clusters and Maturation of the Postsynaptic Apparatus

During development of the NMJ, AChR clusters are formed rapidly after contact between motorneuron and muscle. At birth, clustered AChRs are homogeneously arranged in a flat postsynaptic membrane underneath the nerve, muscle fibers are multiply innervated, and several nerve terminals are intermingled over the postsynaptic apparatus (1). In the first weeks of postnatal development, competitive mechanisms between these

motoneurons lead to synapse elimination. Another aspect of postnatal maturation is the formation of postjunctional folds, leading to segregation of some proteins to become concentrated either at the crests or in the troughs of these folds. AChRs, for example, are localized at the crests, whereas sodium channels are located in the troughs. Using knock-out mice, recent studies indicate that components of the D/UGC, while largely dispensable for initial AChR clustering, are essential both for consolidation and stabilization of AChR clusters as well as for formation of postjunctional folds.

The D/UGC is Required for Stabilization of AChR Clusters and Postnatal Maturation of the Postsynaptic Membrane

The D/UGC links the cytoskeleton of muscle fibers to their extracellular matrix and thereby stabilizes the muscle sarcolemma (100). Differential localization of members of the complex gives rise to synaptic and extrasynaptic variants of the D/UGC. At NMJs, utrophin, β 2-syntrophin, and α -dystrobrevin-1 are highly concentrated and virtually absent from extrasynaptic areas, whereas α -syntrophin, α -dystrobrevin-2, α - and β -dystroglycan, and dystrophin are found both at NMJs and extrasynaptic areas (38,101,102). Additional components of the complex, the sarcoglycans and sarcospan, are found synaptically and extrasynaptically as well (100). Extracellular binding partners for the synaptic D/UGC are agrin and laminin isoforms (laminin chains β 2, α 5, and α 4), which are all concentrated at NMJs (Fig. 1). The largest components of the D/UGC, utrophin and dystrophin, are necessary for the integrity of muscle fibers but largely dispensable for NMJ formation. Utrophin-deficient mice have a surprisingly mild phenotype in that they appear healthy and viable, and have functional NMJs (75,103). The density of synaptic AChRs is slightly reduced, and formation of postjunctional folds after birth is less pronounced. Likewise, NMJs

of *mdx* mice, which lack functional dystrophin, are largely normal, apart from a reduced number of postsynaptic folds. This reduction may result from muscle-fiber necrosis and cycles of regeneration rather than from the absence of dystrophin per se (104,105). These relatively minor effects on morphology of the NMJ in utrophin $-/-$ and *mdx* mice could, in principle, be due to partial compensation between the two proteins. Accordingly, double-deficient mice lacking both dystrophin and utrophin develop a severe muscular dystrophy closely resembling human Duchenne muscular dystrophy (106,107). However, even in these animals, NMJs are remarkably normal, displaying subtle postsynaptic defects such as reduced AChR density and less postsynaptic folds. This suggests that the D/UGC as such is not essential for formation of the NMJ and AChR clusters, but rather for their maturation, in particular, formation of postsynaptic folds.

A component of the D/UGC involved in consolidation and maintenance of AChR clusters and the NMJ is dystroglycan (Table 1). As dystroglycan-deficient mice die at E6.5, before muscles are formed, chimaeric mice were generated by injecting dystroglycan $-/-$ embryonic stem cells into blastocytes of wild-type; these chimeras lack dystroglycan in many striated muscles (108). Dystroglycan-deficient muscles of these mice develop severe muscular dystrophy and, in adult animals, NMJs are fragmented and lack the typical high density of AChRs and AChEsterase. Instead, AChRs are distributed over an increased area, with a coordinate increase in nerve terminal size at these junctions (93). Agrin-induced AChR clustering was analyzed by using myotubes generated from dystroglycan $-/-$ embryonic stem cells. Agrin still induces substantial AChR clustering in these cells, but utrophin, α -dystrobrevin, laminin chains, perlecan, and AChEsterase are not found at these clusters (93,109). As in vivo in chimaeric mice, the area of agrin-induced AChR clusters is increased in dystroglycan $-/-$ myotubes, and the clusters actually consist of many microclusters and disperse rapidly after withdrawal of agrin. Simi-

larly, formation of AChR clusters of normal size is severely compromised in antisense derivatives of C2 myotubes expressing low amounts of dystroglycan (30). As phosphorylation of MuSK and AChR β subunits is unchanged in these antisense cell lines, dystroglycan and the D/UGC as such are not necessary for AChR clustering per se, and dystroglycan is not a coreceptor for MuSK. Overexpression of dystroglycan can decrease AChR clustering at developing *Xenopus* NMJs, however, most likely by adsorbing agrin and preventing it from normal signaling via MuSK (110). Together, these data indicate that dystroglycan is required for integrity of the D/UGC and serves as a membrane anchor to recruit components of the complex to the plasma membrane. Furthermore, dystroglycan is required for consolidation of AChR microclusters into clusters of normal size and for stabilization of AChR aggregates and the NMJ.

Similarly, α -dystrobrevin isoforms are necessary to stabilize AChR clusters (Table 1). Mice deficient for α -dystrobrevin develop muscular dystrophy due to the absence of D/UGC-linked signaling by nNOS, which normally binds to dystrobrevin (111). At the NMJ of these animals, initial synapse formation proceeds relatively normally, but postnatal maturation is affected, because less postsynaptic folds are formed and contain AChRs at crests as well as in troughs, and because AChR-rich areas become fragmented in older animals (109). Synaptic localization of dystrobrevin-binding proteins such as α - and β 2-syntrophin or nNOS, but not other D/UGC components, is reduced in the mutants. Finally, in cultured myotubes, agrin induces normal clustering of AChRs, but these clusters disperse rapidly upon withdrawal of agrin (109). Dystrobrevin is thus necessary for stabilization of AChR clusters in vitro and in vivo and for formation of postsynaptic folds with characteristic segregation of AChRs between crests and troughs. As NMJs in triple knockout mice lacking utrophin, dystrophin, and α -dystrobrevin are not dramatically different from single dystrobrevin knockouts (109), essential synaptic

Table 1
Proteins Necessary for Stabilization of AChR Clusters

Protein	Effect of absence of active protein	Experimental approach	Reference
Dystroglycan	Fragmentation of postsynaptic membrane, muscular dystrophy Rapid dispersal of fragmented AChR clusters	Chimaeric mice lacking dystroglycan in some muscle fibers Withdrawal of agrin from agrin-treated dystroglycan-deficient myotubes	Cote et al., 1999 (108); Jacobson et al., 2001 (93)
Dystrobrevin	Fragmentation of postsynaptic membrane, less postjunctional folds, muscular dystrophy Rapid dispersal of AChR clusters	α -dystrobrevin $-/-$ mice Withdrawal of agrin from agrin-treated α -dystrobrevin $-/-$ myotubes	Grady et al., 1999, 2000 (111,109)
Src and Fyn	Normal NMJ around birth Rapid dispersal of AChR clusters	Src-Fyn-deficient mice Withdrawal of agrin from agrin-treated src $-/-$; fyn $-/-$ myotubes	Smith et al., 2001 (54)
TrkB	Disruption of AChR clusters at the NMJ Disruption of AChR clusters	Adenovirus-mediated expression of dominant-negative TrkB in muscle in mice Adenoviral expression of dominant-negative TrkB in agrin-treated myotubes	Gonzalez et al., 1999 (118)

functions of the D/UGC seem to be mediated by dystrobrevin, probably by localizing its associated signaling molecules to the NMJ.

Finally, absence of α -syntrophin leads to structurally aberrant synapses (112). The isoforms of syntrophin show differential localization at the adult NMJ. Whereas α -syntrophin is found at the crests and in the troughs of postsynaptic folds, β_2 -syntrophin occurs mainly in the troughs; the β_1 isoform is not concentrated at the NMJ (102). In the absence of α -syntrophin, muscle fibers appear healthy histologically and the mice are mobile and fertile (113). NMJs of these animals lack utrophin and

nNOS, have reduced levels of AChRs and AChEsterase and show abnormal postsynaptic folds with fewer openings to the synaptic cleft (112). α -syntrophin thus mediates localization of utrophin and nNOS to the NMJ, and the similarities in NMJs between α -syntrophin- and utrophin-deficient mice suggest that α -syntrophin functions to localize utrophin at the synapse, which organizes postsynaptic fold formation.

Together, studies on knockouts of components of the D/UGC show that this complex is largely dispensable for initial formation of AChR clusters and the NMJ. Instead, the com-

plex is mainly required for postnatal maturation of the postsynaptic membrane as well as for stabilization of AChR clusters.

Tyrosine Kinases Are Required for Stabilization of Agrin-Induced AChR Clusters

Three lines of evidence have recently indicated that, in addition to the D/UGC, tyrosine kinases are important for the stability of agrin-induced AChR clusters. First, upon removal of agrin, the kinase inhibitors herbimycin and staurosporine disperse pre-formed AChR clusters in myotubes (56), showing that tyrosine kinase activity is necessary to maintain AChR clusters. This activity most likely originates from another kinase than MuSK, because staurosporine does not affect the autophosphorylation of MuSK (62).

Second, Src and Fyn mediate stabilization of AChR clusters, because in myotubes cultured from Src-Fyn-doubly deficient mice, agrin induces normal phosphorylation and aggregation of AChRs, but these clusters disperse rapidly after withdrawal of agrin (54). Src and Fyn are thus dispensable for AChR clustering, but essential for cluster stabilization. PP1 and PP2, inhibitors of Src-family kinases, do not decrease the stability of AChR aggregates, suggesting that cluster stabilization does not primarily require the kinase activities of Src and Fyn, but rather their presence as adapters, by mediating protein interactions (54; Table 1). These findings reveal interesting parallels to focal adhesion sites, where Src, independent of its kinase activity, can recruit and activate other proteins such as focal adhesion kinase (FAK) (114,115). Furthermore, multiple tyrosine kinases, including Src, FAK, and Pyk2, can complement each other to achieve optimal adhesion on fibronectin (116), raising the possibility that interaction of multiple kinases may regulate interactions between the postsynaptic membrane and cytoskeleton elements at the NMJ, thereby stabilizing AChR clusters. In support of this, several cytoskeletal proteins such as

talin, paxillin, and α -fodrin can be tyrosine-phosphorylated and are concentrated at the mammalian NMJ or in Torpedo synaptic membranes (117).

Third, TrkB is also involved in cluster stabilization. Disruption of TrkB-mediated signaling, by overexpression of a dominant-negative form of TrkB, decreases the stability of AChR clusters *in vivo* and in agrin-treated cultured myotubes (Table 1; 118). TrkB is localized to the postsynaptic membrane in muscle and can associate with Fyn in cortical neurons (118,119), raising the possibility that TrkB signaling mediated by Src-family kinases stabilizes AChR clusters in muscle. The ligand for TrkB mediating this effect is not known. BDNF, a prominent ligand of TrkB, and NT-4 inhibit agrin-induced AChR clustering in cultured myotubes (120). TrkB may thus exert two different effects at the NMJ, depending on the stage of NMJ differentiation. Early in postsynaptic assembly, it may—stimulated by BDNF—negatively regulate AChR clustering, while later it stabilizes the postsynaptic membrane.

Together, these studies show that several tyrosine kinases, at least Src, Fyn, and TrkB, are involved in stabilization of AChR clusters. The mechanisms for formation vs stabilization of AChR clusters are thus different, both in terms of structural proteins, as reflected by the D/UGC, as well as in their requirement for signaling proteins, as shown in the case of Src, Fyn, and TrkB. It remains to be determined whether and how tyrosine kinases and the D/UGC collaborate to achieve optimal stabilization. One possible link is dystrobrevin, which is a substrate for tyrosine kinases and strongly tyrosine-phosphorylated in Torpedo electric organ (121). Although α dystrobrevin is neither required for early steps in agrin-induced AChR clustering nor tyrosine-phosphorylated by agrin stimulation (101), its phosphorylation may play a role in stabilization of AChR clusters at the developing NMJ. This would be consistent with the striking similarity in dispersal of AChR clusters in dystrobrevin $-/-$ and Src-Fyn-deficient myotubes, as

well as in muscle cells overexpressing dominant-negative TrkB (Table 1).

A Role of AChRs in Postsynaptic Stabilization

During postnatal NMJ development, the composition of AChRs changes, as the embryonic γ subunit is replaced by the adult ϵ chain. By deleting the ϵ gene in mouse, the role of AChRs in this phase of NMJ development was analyzed. In $\epsilon^{-/-}$ mice, the high postsynaptic density of AChRs is gradually reduced in the first weeks after birth, as these animals fail to synthesize adult-type AChRs (122,123). Consequently, the mice develop gradual muscle weakness and atrophy, and die prematurely. Along with the reduced AChR density, postjunctional folds disappear, in such a way that the postsynaptic membrane, although rich in folds at 20 d, is flattened out by 60 d of age (124). Further, postsynaptic components such as rapsyn, utrophin, MuSK, erbB4, phosphotyrosine, and dystrobrevin are reduced as the AChR density decreases (122,124). Interestingly, rapsyn disappears strictly in parallel with AChRs, while utrophin and dystrobrevin lag behind (124). Thus, ϵ -containing AChRs are required for synaptic localization of rapsyn and maintenance of the postsynaptic apparatus at the postnatal NMJ, including postjunctional folds.

In addition, activity through the AChR is necessary to maintain its high synaptic density (125). In adult mice, AChRs are highly metabolically stabilized, displaying a half-life of about 14 d. Immediately after blocking AChRs by α -bungarotoxin (BTX), the half-life is reduced to less than a day, resulting in rapid turnover of postsynaptic AChRs and a decrease in the density of postsynaptic AChRs. This decrease in half-life can be stopped by direct stimulation of muscle or restoration of synaptic transmission (125). These data indicate that AChR activation is required for its synaptic stability and that AChRs serve structural roles in maintaining the postsynaptic apparatus. AChRs thus appear to be necessary

for both formation and maintenance of the postsynaptic apparatus at the NMJ.

Nicotinic Receptors in the Nervous System

Multiple classes of neurotransmitter receptors localized at the synapse of a neuron and different setup of synaptic excitability of target cells suggest that synaptic features, which have been characterized in the muscle, might not simply be translated into the nervous system. Differently from the muscle type, nicotinic receptors in the nervous system are characterized by high Ca^{2+} permeability and considerable diversity of their subunits. So far, nine different α ($\alpha_{2,3,4,5,6,7,8,9,10}$) and three different β subunits ($\beta_{2,3,4}$) have been identified in vertebrates, which are encoded by distinct genes (126–128). Differential splicing of some of the genes further extends this already existing list of clones (129).

Functional subtypes of receptors that can be generated from different arrays of subunits are equally diverse although they all appear to form a pentameric structure (127,130). α_7 , α_8 , and α_9 subunits form a homo-oligomeric complex when expressed as a single subunit and comprise the α -bungarotoxin (BTX)-binding class of AChRs. The α_7 subunit is one of the most abundant subunits that are expressed in the vertebrate nervous system and α_7 receptors are characterized by high Ca^{2+} permeability that is comparable to NMDA receptor channels (131,132). Other subunits require α or β to be functional as a hetero-oligomer. Prominent examples are $\alpha_4\beta_2$, which is the most abundant subtype in the brain and forms high-affinity nicotine binding sites, and ganglionic subtypes of receptors containing α_3 , α_5 , β_2 , and β_4 . Expression of multiple subunit genes in the same neuronal population suggests that, in reality, the profile of subtypes of neuronal receptors is much more complex (126,133,134).

Mechanisms of cholinergic synaptogenesis in the nervous system are largely elusive com-

pared to the NMJ and have been mainly investigated in the PNS. Studies about the CNS synapses have been limited, mainly due to the relatively low expression level of nicotinic receptors in limited areas of the CNS. Thus, compared to other receptor systems, very little information is available about how interneuronal cholinergic synapses in the CNS are built up and maintained. In the following parts, studies that have been made so far for characterizing nicotinic synapses in the PNS and CNS are summarized, and we attempt to postulate postsynaptic components of these synapses, mainly based on the information about the molecules identified at the NMJ.

Cholinergic Synapses in the Peripheral Nervous System

Functional cholinergic synapses have been identified in a number of peripheral neurons from various species, e.g., superior cervical, ciliary, and sympathetic ganglia (135–137). In the PNS, nicotinic receptors mediate excitatory synaptic transmission as major neurotransmitter receptors, although a presynaptic function, regulating the release of neurotransmitters, has also been demonstrated (134,138).

Postsynaptic structures and functional aspects of interneuronal cholinergic synapses are best-characterized in chick ciliary ganglia where developmental and innervation profiles have been well-investigated. Chick ciliary ganglion neurons express α_3 , α_5 , β_2 , β_4 , and α_7 subunits and, thus far, two major subtypes of receptors differing in their subunit-compositions and also functional properties have been identified, that is, the α -BTX sensitive component comprised of α_7 subunits and MAb35-immunoreactive receptors of which at least two classes exist depending on the presence of β_2 subunits, e.g., $\alpha_3\alpha_5\beta_2\beta_4$ and $\alpha_3\alpha_5\beta_4$ (139–141). In the chick ciliary ganglion, α_7 and mAb35-AChRs show interesting differences in their localizations with respect to synaptic sites. Whereas clusters of α_3/α_5 subunit-containing

ganglionic receptors are found at extrasynaptic sites in addition to postsynaptic densities, those of α_7 -containing receptors that represent the most abundant population of ganglionic receptors, are exclusively localized on perisynaptic somatic spines (135,142). By ultrastructural analysis, synaptic MAb35-AChRs were localized to the postsynaptic density (PSD) opposing presynaptic vesicle release sites. Perisynaptic α_7 receptors on spines, interestingly, are present in close proximity to the potential release sites overlaid with vesicles (142,143). Although α_7 subunits are excluded from the synapse, they carry majority of the synaptic current and it appears that they are important for rapid and reliable synaptic firing (144,145).

What mechanisms could be involved in anchoring/targeting of these receptors at their respective sites? Recent studies show that in chick ciliary ganglia actin filaments are colocalized with α_7 receptors and are active components for maintaining α_7 receptor-clusters in the postsynaptic membrane, since depolymerization of actin filaments disperses these clusters and also accelerates agonist-challenged rundown of the α_7 channel function (142,146). In addition to association, either directly or indirectly, with f-actin, anchoring perisynaptic α_7 receptors on somatic spines appears to involve other mechanisms, since these receptors are resistant to extraction by nonionic detergents even after treatment with actin-depolymerizing agents (142). A recent study has demonstrated that α_7 receptor clusters are tightly linked to membrane lipid rafts. As shown by treatments that disperse rafts, the integrity of these rafts is required to maintain α_7 clusters, independently of f-actin, thus offering an explanation for an additional anchoring mechanism (147). Lipid rafts are rich in Src-family kinases and other signaling molecules (148). This suggests that Src and Fyn might play a role in maintaining α_7 clusters and this could be an interesting parallel to the NMJ where these kinases are necessary to maintain AChR clusters (54). In contrast, ganglionic MAb35-reactive receptors, although partly found on somatic spines along with α_7 , appear

to require different mechanisms for localization in the plasma membrane since their functional integrity is not affected by repeated applications of nicotine and they are also readily solubilized by nonionic detergents (142,146). These receptors are targeted to the postsynaptic density by a signal in the cytoplasmic portion of the α_3 subunit: studies using chimeric receptor constructs showed that the cytoplasmic loop of the α_3 subunit mediates postsynaptic localization, whereas the corresponding region of α_5 lacks such targeting information (149). Another candidate for localization of neuronal AChRs is rapsyn, based on its importance at the NMJ. Although rapsyn causes clustering and detergent-insolubility of various nicotinic receptors upon expression in heterologous fibroblasts, it is not required for clustering of ganglionic AChRs in the superior cervical ganglion neurons, as shown in rapsyn $-/-$ mice (*see below*;137). Further, although mRNA is present, postsynaptic rapsyn immunoreactivity was not detected in this ganglion nor in chick ciliary ganglia, indicating that rapsyn might play a different role, if any at all, in the nervous system.

Another complexity of interneuronal cholinergic synapses originates from the presence of glycine receptor-microdomains that are associated with the postsynaptic densities of chick ciliary neurons (143). Chick gephyrin specifically colocalizes with these glycine receptors, but not with MAb35-reactive AChRs. Segregation of different neurotransmitter receptors and different subtypes of receptors into mosaics of domains, using cognate clustering/anchoring molecules, thus, may be another mechanism for the neuron to fine-tune synaptic transmission.

Finally, extrasynaptic clusters of nicotinic receptors, in addition to clustering at the postsynaptic sites, have also been observed in the mouse superior cervical ganglion and the frog cardiac ganglion (137,150), which raises the possibility that nonsynaptic localization of nicotinic receptors, yet taking part in a significant portion of synaptic transmission, might be a general feature of peripheral nicotinic

synapses. Although NMDA receptors in cultured hippocampal cells were found clustered extrasynaptically in early neuronal development, molecular explanations have not been provided. Further, clustering of ligand-gated ion channel receptors outside of postsynaptic sites in mature neurons has not been ubiquitously found and investigated (151).

Cholinergic Synapses in the Central Nervous System: An Overview

Autoradiographic and subunit mapping studies using various ligands, antibodies, and cRNA probes have generated useful information about the distribution of nicotinic receptors and cholinergic synapses in the CNS (130). Although these studies implicated cholinergic functions in the brain, unequivocal identification of cholinergic synapses carrying nicotinic receptors as the mediator of major synaptic transmission turned out to be difficult and has been limited to few examples (152). This has been attributed to the presynaptic role of nicotinic receptors as a primary function and examples are well-documented by numerous studies where presynaptic functions are shared by a variety of neurotransmitters, e.g., glutamate, dopamine, GABA, noradrenaline, and ACh itself (134). However, a collection of recently made studies, mostly using immunocytochemical and refined-electrophysiological analysis, begun to generate accumulating evidence for the postsynaptic cholinergic function, indicating that nicotinic receptors can act as the major carrier of synaptic current. In the following section, studies that provided direct or indirect evidence for the role of nicotinic receptors in mediating rapid synaptic transmission are summarized and discussed.

Immunocytochemical Characterization of Cholinergic Synapses

α_7 , α_4 , and β_2 subunits are widely expressed species of subunits in the brain, while expression profiles of other subunits are more limited

(153–155). Some of the areas where these subunits are abundantly expressed served as good model system for investigating postsynaptic aspects of cholinergic synapses. However, a number of neurons express multiple subunits simultaneously and the question of how these are organized as synaptic responsive elements at the synapse is elusive and remains to be investigated.

Strong α_4 immunoreactivity has been mapped to tyrosine hydroxylase (TH)-positive dopaminergic neurons in substantia nigra pars compacta (SNpc) and ventral tegmental area, of which cell bodies and dendrites are innervated by neurons from pedunculo pontine tegmental nuclei (PPN) and the laterodorsal tegmental nucleus (156–158). Postsynaptic nicotinic responses that can be evoked by stimulating inputs from PPN and also excitability of dopaminergic neurons in SNpc by application of nicotine indicate that nicotinic receptors directly mediate synaptic transmission in these neurons (159,160). Dopaminergic neurons express multiple nicotinic subunits and studies from different lines suggest that the $\alpha_4\beta_2$ combination of receptors among others are likely candidates for functional nicotinic receptors mediating fast synaptic transmission (161–164). Light microscopic and ultrastructural analysis revealed some features of subcellular distribution of α_4 subunits. α_4 -immunoreactivity was associated with cell perikarya and dendritic shafts, but not with spines (158,160). Labeling of some postsynaptic structures was evident as assessed by electron microscopic analysis, but some nonsynaptic labeling was also identified. Further, a detailed study using postembedding immunogold procedures demonstrated clear labeling of α_4 at the PSD, although the frequency of the labeling was low (158).

So far, studies that demonstrated association of nicotinic receptor subunits with the PSD in the CNS have been limited to α_4 subunits. Although α_4 subunits decorate the PSD at very low frequency in dopaminergic neurons of the substantia nigra, PSD association has been demonstrated more convincingly in other brain areas such as the paraventricular nucleus

of the hypothalamus and pyramidal cells in layer 5 of the rat cortex (165,166). The common theme arising from these studies is the distribution of α_4 subunits in the cell body and proximal rather than distal parts of dendrites. It is notable that β_2 subunits, which preferentially assemble with α_4 subunits and form high-affinity nicotine binding sites, also exclusively label perikarya with rare synaptic labeling in the brain as judged by electron microscopic analysis (154).

Another example for fast synaptic nicotinic responses is found in the chick lateral spiriform nucleus (SpL) that receives cholinergic projections from the nucleus semilunaris. Nicotinic synapses have been identified by the double-label immunofluorescence technique using antibodies to choline acetyltransferase (ChAT) and MAb35 (167). ChAT-positive immunoreactivity was associated with that of MAb35 more preferentially in the fibers lateral to SpL than in neurons in the nucleus, and colocalization was pronounced in dendrites but not on the neuronal soma of these neurons. The clusters of MAb35-immunoreactive nicotinic receptors were not frequently colocalized with presynaptic vesicle markers, suggesting that most of clusters are of nonsynaptic origins (168). However, stimulation of cholinergic afferents to SpL neurons generated nicotinic excitatory postsynaptic potentials (EPSPs) that can evoke action potentials (167). These responses were not sensitive to antagonists for α_7 receptors and might be mediated by $\alpha_5\beta_2$ subunit-containing receptors since they are expressed by more than 90% of the neurons in the SpL (168). Thus, nicotinic receptors in SpL neurons are primarily concentrated at extrasynaptic sites, and yet they participate in eliciting the postsynaptic response.

α_7 subunits that constitute α -BTX binding sites are enriched in the hippocampus and some nuclei of the brain stem (155,161). Morphological analysis suggests postsynaptic roles of these receptors since there was no significant axon terminal staining. The subcellular staining pattern of α_7 varied across different neuronal groups in terms of the extent of dendritic label-

ing, since all dendritic trees were labeled in some cases and, in others, only very proximal portion of dendrites were labeled. Even with frequent labeling across the brain, functional α_7 receptors mediating fast synaptic transmission have been conclusively identified only in interneurons of the hippocampus (*see below*). In contrast with the PNS, clustered postsynaptic patch type of immunostaining was not observed at least in tissue sections. However, in a separate *in vitro* study using cultured neurons from the hippocampus, clusters of α_7 subunits were observed along the soma and dendrites and they were colocalized with presynaptic markers (169,170). Since most of these cultured neurons are pyramidal cells, this has been interpreted as segregation of α_7 subunits into presynaptic terminals of these neurons, regulating the release of glutamate. A physiological parallel is provided by the study where α_7 receptors enhanced the release of glutamate at the mossy fiber-CA3 pyramidal cell synapses (171). In comparison to α_7 , β_2 subunits were localized in the cell body in a diffuse way in cultured hippocampal cells, suggesting specific segregation of different subtypes of receptors into different subcellular compartments. The discrepancy between studies using cultured cells and tissue sections for aggregation of α_7 immunoreactivity could be due to the system used, that is, *in vivo* vs *in vitro*, and awaits further detailed characterization.

Functional Evidence for Cholinergic Transmission

Direct isolation of postsynaptic responses mediated by nicotinic receptors was made possible by recent studies that employed detailed electrophysiological analysis. Thereby, functional cholinergic synapses were demonstrated in various principal cells and interneurons of different brain regions. Spontaneous or nicotinic agonist-mediated postsynaptic responses were observed in a number of brain areas, e.g., neurons in the interpeduncular nucleus and medial habenula (172,173), nucleus ambiguus of the brain stem (174), medial vestibular

nucleus (175), and low-threshold spike cells in layer 5 of the rat visual cortex (176).

Fast cholinergic synaptic transmission with interesting physiological implications was found in the developing ferret visual cortex (177), apart from peripheral synapses of retinal ganglion cells (178). Some principal cells and interneurons in the developing ferret visual cortex displayed spontaneous EPSCs and also stimulation of thalamic afferents elicited cholinergic postsynaptic responses. Synaptic currents were insensitive to α -BTX, excluding the α_7 class of receptors. Interestingly, an increase in this widespread nicotinic synaptic transmission coincided with the period of eye-opening, suggesting a role of nicotinic receptors in synaptogenic events or circuit remodeling in this period.

In addition to these studies, with the importance of the hippocampus as a structure mediating learning and memory and with the aid of its relatively well-defined circuitry, much attention has been drawn to the synaptic functions of nicotinic receptors in the hippocampus, revealing detailed aspects of cholinergic functions and also receptor subtypes mediating those. Pyramidal cells and interneurons of the hippocampus receive cholinergic innervation from the medial septum-diagonal band complex of the basal forebrain (179–181). Although α_7 subtype-mediated synaptic currents have been reported in pyramidal neurons of the CA1 area in some cases, generally they appear not to carry functional nicotinic synapses (182–184). Interneurons in the CA1 field, however, express multiple components of nicotinic responses that differ in kinetic properties and pharmacological profiles. Further, different populations of interneurons express different functional subtypes and some express multiple classes of receptors, revealing complexity of the cholinergic synapses. The main, rapidly depolarizing synaptic response in CA1 interneurons is mediated by α_7 receptors, whereas the rest of the slowly depolarizing components are mediated by other classes comprising α_2 – α_5 and β_2 – β_4 , which overall can be grouped into four pharmacologically differ-

ent subtypes (169,184–187). In support of electrophysiological observations, α -BTX binding sites were found at the underlying synapses on interneuronal somata where cholinergic boutons appear to synapse (179,188). Excitation of these interneurons will influence the activity of a large number of principal cells, thereby coordinating overall hippocampal output (138).

Another intriguing physiological significance of cholinergic synapses in the hippocampus is provided by the study where activation of α_7 receptors enhances the LTP induction process in the CA1 shaffer collateral/commissural pathway (189).

Further approaches, combining electrophysiology with immunocytochemistry using high-affinity antibodies specific for nicotinic subunits, will help to understand better the location and postsynaptic architecture of cholinergic synapses in the CNS.

Postsynaptic Assembly: From the NMJ to the Nervous System

Anchoring and clustering of neurotransmitter receptors at functional sites are a critical task for neurons to perform efficient synaptic transmission. A number of synaptic components including clustering molecules have been identified for other neurotransmitter receptors (190,191). Especially, existence of multiple isoforms for some synaptic molecules such as the PSD95 family of proteins further impose complicated features of the CNS synapses. However, to date, very little information is available for the question of how cholinergic synapses in the nervous system are constructed and maintained, especially in the CNS. One plausible hypothesis would be that structural and signaling components, which have been characterized at the NMJ, are also involved in the assembly of the postsynaptic scaffold in the nervous system. Indeed, many molecules of the NMJ are also expressed in the PNS and CNS, and some, such as the D/UGC, appear to assemble into functional complexes

in a similar way as in the muscle (192,193). However, except rapsyn, the relationship between these proteins and nicotinic synapses has not been systematically investigated so far. Another possibility is that interneuronal cholinergic synapses are associated with as yet unidentified postsynaptic scaffolding proteins. For example, gephyrin, which is involved in clustering of glycine and GABA_A receptors, does not appear to be a synaptic component of cholinergic synapses (143).

Agrin May Play a Role in Postsynaptic Assembly at CNS Synapses

Several studies have addressed the question of whether agrin, in analogy to its crucial role at the NMJ, plays a similar role in the formation of CNS synapses, however, the outcome is unclear at present. Agrin is widely expressed in the CNS, with prominent levels in the hippocampus and olfactory bulb (194). In agrin-deficient mice, synaptic localization and clustering of glutamate and GABA_A receptors were not affected in the hippocampus, neither in tissue sections nor in cultured neurons (195). Similarly, glutamatergic and GABAergic synapses are formed normally in cultured cortical neurons derived from agrin $-/-$ mice, as was tested using immunocytochemical and functional analysis (196). In contrast, in *in vitro* studies using hippocampal cultured neurons, suppressing the expression of agrin by treatment with antisense oligonucleotides or agrin-antibodies resulted in the formation of fewer synapses that were functionally inefficient (197,198). The discrepancy among these studies might be due to compensatory synaptogenic mechanisms *in vivo* operating in the agrin-deficient mice. Some indirect lines of evidence also suggest that agrin might be involved in the formation of the interneuronal synapses, including postsynaptic assembly. Agrin expression in the developing cortex coincide with the formation of thalamocortical and intracortical synapses (199). Also, during development of the retina, agrin is redistributed from an initial diffused state to a punctate pattern in the inner and outer plexiform

layers, where it is concentrated within the synaptic clefts (200,201). Moreover, in cultured retinal neurons, clusters of agrin colocalize at synaptic sites with gephyrin, one of the markers of inhibitory synapses, and purified retinal agrin induces AChR clustering in cultured myotubes (202). These results suggest a role of agrin in postsynaptic assembly, including receptor clustering, at synapses in the retina. However, in the CNS, studies about localization and expression of agrin have so far not investigated the presence of agrin at synapses in detail. Recently, a new form of agrin in which the NtA domain of the N-terminus is replaced by a cell surface-associating transmembrane domain was isolated from murine species and was found to be expressed mainly in the brain (88,203). This isoform was also able to cluster AChRs in myotube cultures, suggesting that it might play a role in clustering of nicotinic or other receptors at the CNS interneuronal synapses.

With respect to cholinergic synapses, agrin is expressed in neurons of the chick ciliary ganglion (204). Agrin is also expressed by the innervating cholinergic neurons of the Edinger-Westphal nucleus: this expression, along with agrin expression in ganglionic neurons, coincides with the periods of synapse formation and clustering of AChRs (205). In tissue culture, agrin from agrin-expressing Chinese Hamster Ovary (CHO) cells arrests the neurite outgrowth of ciliary neurons and induces aggregation of the synaptic vesicle protein, synaptotagmin, suggesting an effect on presynaptic differentiation (206). Therefore, agrin may play roles both in pre- and postsynaptic differentiation at cholinergic synapses in the ciliary ganglion, reminiscent of its roles at the vertebrate NMJ.

Rapsyn Is Not Required for Clustering of Ganglionic AChRs

Rapsyn, which is essential for the formation of AChR clusters at the NMJ, can cluster neuronal nicotinic receptors of α_7 , α_3/β_2 , α_3/β_4 , or α_4/β_2 combinations and the α_1 , β_1 , γ_2 -GABA_A

receptor complex in heterologous cells (137,207,208). However, evidence supporting rapsyn as a clustering molecule in neurons is lacking. Rapsyn mRNAs, including several shorter splice variants that are not active in clustering of neuronal AChRs (differently from the full-length form), are readily detected in chick ciliary and mouse superior cervical ganglia, while the expression level in the brain is very low (137,208,209). In these studies, however, rapsyn immunoreactivity was not detected in ganglionic neurons (137,208). Most importantly, clusters of ganglionic AChRs containing α_5 and β_2 subunits are present in normal number, size and densities in superior cervical ganglia of mice lacking rapsyn, showing that rapsyn is not required for clustering of ganglionic AChRs (137). The reason for the expression of rapsyn transcripts without proteins in neurons is not clear, but could imply that, in the nervous system, rapsyn might play a different role than clustering.

Components of the D/UGC Are Associated with Interneuronal Synapses in the PNS and CNS

Dystrophin, the major component of the D/UGC in muscle, is expressed in neurons of the PNS and CNS. Immunohistochemical and biochemical analysis demonstrated that dystrophin is associated with the PSDs in mouse superior cervical ganglia, some neurons of the cortex and cerebellar Purkinje cells, and is found along the soma and dendrites as clusters in the CNS neurons (210–213). In addition to the muscle type, a number of short forms of dystrophin-related gene products that still carry critical interaction domains with other D/UGC proteins have been identified in the nervous system, although functional roles of those are not clear (192,212,214,215). There exists some evidence that these different gene products are localized at different synaptic sites (212). Synaptic functions of dystrophin have been implicated in some studies. Clusters of α_1 and β_2 subunits of GABA_A receptors were colocalized with dystrophin immunoreactivity in

the hippocampus and cerebellum and they were markedly reduced in dystrophin-deficient *mdx* mice, while there was no change in the number of gephyrin clusters (213). This suggests that dystrophin is involved in the synaptic localization of at least some subpopulations of GABA_A receptors. A possible link between synaptic AChRs and the D/UGC is found in the following studies. Ganglionic axotomy generated a coinciding reversible decrease of immunoreactivity of β -dystroglycan, dystrophin, and α_3 AChR subunits in the mouse superior cervical ganglion, which preceded the decrease in the number of intraganglionic synapses (216). In addition, synaptic localization of α_3 AChR subunits, α - and β -dystroglycan was reduced in the superior cervical ganglion of *mdx* mice (which lack full-length dystrophin but still express short dystrophin isoforms). These findings suggest that full-length dystrophin is involved in the synaptic localization of α_3 , by associating with these receptors in the same synaptic complex (217). Therefore, dystrophin appears to be associated with synaptic GABA_A and nicotinic receptors even though the role as a synaptic component is not entirely clear.

β -dystrobrevin and α -dystrobrevin-1, members of the dystrobrevin gene family, are mainly expressed in neurons and glia, respectively (215). β -dystrobrevin is enriched at post-synaptic densities of major neurons in the brain and its expression pattern resembles that of dystrophin. β -dystrobrevin can be isolated together with syntrophin and dystrophins from the brain, indicating the presence of a complex similar to the muscle D/UGC (215,218). However, the molecular architecture of these protein complexes appears to be different between the muscle and the brain since, in dystrophin-deficient *mdx* mice, dystrobrevin-syntrophin complexes are still formed in the brain, whereas their assembly is disrupted in the muscle (215). The presence of β -dystrobrevin in the brain, but not in the muscle, further illustrates the structural difference of the muscle and brain forms of the D/UGC (214).

Syntrophin, another D/UGC component, also exists as multiple forms encoded by distinct genes. Two novel variants of human origin, γ_1 and γ_2 isoforms, have been cloned recently and are expressed in neurons of the brain, mainly the cortex and hippocampus, decorating the soma and proximal dendrites (219). Other syntrophins (α_1 and β_2) are also expressed in the hippocampus and their mRNAs colocalize with those of dystrophins (192). All of these syntrophin isoforms can bind to dystrophin as shown by in vitro assays (219,220).

Dystroglycans and utrophin also are expressed in neurons in an analogous manner to the aforementioned D/UGC proteins, but they appear to also decorate some microvasculature structures and glia (221,222). Utrophin tends to label neurons differently from dystrophin in a nonoverlapping manner, not only on the cellular but also subcellular levels, suggesting possibly different functional roles between them (223).

Although these studies indicate that components of the muscle D/UGC are widely expressed in the brain, some at synaptic sites, and can form similar complexes, functional roles of these proteins with respect to CNS synapse formation have not been unequivocally established. So far, sodium channels have been isolated as a binding partner for syntrophin, therefore, indicating that components of the D/UGC can be directly associated with neuronal ion channels (224). It remains to be determined whether direct association with neurotransmitter receptors also underlies the suggested role of dystrophin at central GABAergic and peripheral cholinergic synapses (see above).

Possible Roles of Presynaptic Input and the Cytoskeleton in Maintaining Synaptic AChRs

Maintenance of the high synaptic density of AChRs at the NMJ requires activation of these receptors (125). Some studies suggest that this

might also be the case for cholinergic synapses in the PNS. In frog cardiac ganglion neurons, large/dense clusters of AChRs at synaptic sites were dispersed into small clusters over the cell surface by denervation (135). However, synaptic activity-dependent activation of receptors does not appear to be necessary for the development of some classes of receptor clusters, as has been mainly investigated by in vitro assays using cultured neurons. Although glutamatergic and glycinergic inputs are necessary for clustering of AMPA and glycine receptors, respectively, clustering of NMDA and GABA_A receptors does not require cognate presynaptic input (225–228). Therefore it appears that organization of different classes of synapses requires not only different synaptic modules, but also different modes of activity-based regulation. A close correlate to the NMJ is found in another aspect of the PNS, that is, in chick ciliary ganglion neurons, presynaptic input induces the expression of AChR mRNAs during synaptogenesis and also maintains their expression (229,230).

The cytoskeleton is an important component for anchoring neuronal receptors at synaptic sites, and the regulation of cytoskeletal assembly serves as a mechanism for stabilizing or destabilizing synaptic receptor-complexes for a number of neuronal receptors. So far, tubulin and actin have been identified as cytoskeletal elements that regulate clustering events of GABA_A and glutamate receptors (191,231,232). Interaction of receptors with the cytoskeleton occurs mostly through clustering molecules such as gephyrin, GABARAP, and PSD95, although direct interactions also occur (191,233–235). Evidence for interaction of neuronal nicotinic receptors with the cytoskeleton is limited to ganglionic α_7 receptors (*see above*). Although anchoring phenomena at postsynaptic densities found for ganglionic AChR subunits as well as for CNS subunits such as α_4 , suggest involvement of the cytoskeleton for synaptic localization of these receptors, there has been no study supporting this hypothesis.

Conclusions

Mechanisms of postsynaptic assembly that were established at the NMJ potentially serve as good models for understanding synaptic development of interneuronal synapses. Whether development of the neuronal cholinergic synapses requires some of the molecules that have been identified at the NMJ, or whether it depends on unique build-up machinery, is an intriguing open question. For example, rapsyn, an essential component for clustering of AChRs at the NMJ, is dispensable for clustering of neuronal nicotinic receptors. However, actin filaments are required for maintaining clusters of both muscle and neuronal AChRs and some D/UGC proteins are associated with peripheral cholinergic synapses. Considering different classes of neuronal receptors that are segregated in a given synapse, as demonstrated in peripheral ganglia, it is also tempting to question how these unique synaptic structures and functions are formed and maintained.

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